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Impact of the Hard-to-Cook Phenomenon on Phenolic Antioxidants in Dry Beans (*Phaseolus vulgaris*)

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Epidemiological studies have established a link between consumption of dry beans and lower incidence of degenerative diseases. This relationship is attributed in part to properties of natural antioxidants present in beans. The objective of this study was to determine if the hard-to-cook (HTC) phenomenon in beans had a negative effect on the content of free and bound phenolic antioxidants and antioxidant capacity. Folin−Ciocalteu, Trolox equivalent antioxidant capacity, and HPLC methods were used to quantify the content of phenolic acids and antioxidant capacity. Results showed that the HTC phenomenon did not equally affect the content and antioxidant capacity of phenolic acids in different bean cultivars. Black beans were most affected, the contents of free and acid hydrolyzable phenolic acids being reduced by 35 and 36%, respectively, and the antioxidant activity by 18 and 25%, respectively. This study showed that the HTC phenomenon affected a potential nutritive characteristic of dry beans.

KEYWORDS: Common beans; *Phaseolus vulgaris*; phenolic acids; antioxidant activity; hard-to-cook phenomenon

INTRODUCTION

Epidemiological and demographic studies indicate that populations with the greatest consumption of beans have a reduced risk of mortality from breast, prostate, and colon cancers (*1*). Several authors have mentioned that beans contain potentially bioactive microconstituents, such as phenolic acids, that have demonstrated anticarcinogenic and antioxidant properties in both in vitro and animal models (*2*−*9*). It is generally believed that phenolic acids can be important biological antioxidants by scavenging free radicals and reactive oxygen species, chelating metal catalysts, activating antioxidant enzymes, and inhibiting oxidases that lead to degenerative diseases (*10, 11*). Common beans (*Phaseolus vulgaris*), especially black cultivars, are a rich source of antioxidants and may provide health benefits similar to those of some common fruits and vegetables (*3*). Work on beans has focused on the antinutritional effects of seed coat polyphenolic compounds such as condensed tannins; only a few studies exist on the content and beneficial effects of the wide array of phenolic compounds found in beans (*2, 12*).

Phenolic acids and their derivatives are widely distributed in legumes and can be present in either free (extractable) and/or bound (both extractable and nonextractable) form (*13*). To date, work on the antioxidant activity of beans has focused mainly on free forms of phenolic acids (*2, 10, 11, 14*) because those are believed to be most bioavailable to humans. However, the bound phenolic acids may become accessible after processing of the food products or through the action of microorganisms in the lower gut.

The benefit of the phenolic acids present in beans against oxidative stress conditions in the human body could be affected by a hardening phenomenon, which is known as the hard-to-cook (HTC) defect. The HTC phenomenon is characterized by extended cooking times following storage under high temperature and high relative humidity (*15*−*17*). Beans with this defect are less acceptable to consumers, have lower nutritional value, and require more energy to cook (*13*). Information about the effect of typical conditions of storage in Latin America or Africa on the antioxidant capacity may help to improve future postharvesting activities to maintain this important characteristic of beans.

Several theories have been suggested to explain this hardening defect in beans, such as (a) lipid oxidation and/or polymerization, (b) formation of insoluble pectates, and (c) lignification of middle lamella (*18*). Changes in the phenolic acids content and profile in HTC beans also have been shown (*13, 16, 19*). However, current studies used one or two cultivars and did not compare cultivars of different seed colors (*2, 10*).

The objectives of this study were first to identify differences in the content of free and bound (alkaline and acid hydrolyzable) phenolic acids between normal and HTC beans and, second, to establish whether or not the HTC phenomenon affects the antioxidant activity of beans and the content of specific phenolic acids.

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MATERIALS AND METHODS

Chemicals. Methanol, hydrochloric acid, sodium hydroxide, acetic acid, and sodium carbonate were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Folin−Ciocalteu reagent, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), HPLC grade ferulic (FA), gallic, protocatechuic, caffeic, syringic, *p*-coumaric, and sinapic acids, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), and potassium persulfate were purchased from Sigma-Aldrich (St. Louis, MO). Ammonium chloride and HPLC grade methanol were purchased from Fisher Scientific (Somerville, NJ).

Bean Cultivars and Sample Preparation. Three cultivars of beans were obtained from the Central Bean Co. (Quincy, WA): Seahawk, Lebaron, and T-39 (white, red, and black cultivars, respectively). Beans of the same cultivar were from the same production lot and harvested during the fall of the year before the experiment was conducted. They were stored at room conditions (21 °C and 50% relative humidity) for 5 months before the storage study was begun. Some studies have reported a positive correlation between the antioxidant activity of fruits and vegetables and the content of anthocyanins (flavonoids pigments in the seed coat) (*3, 9*). Therefore, beans of three different seed coat colors were selected for this study, because it was assumed that legumes with the highest polyphenolic content are dark varieties such as black and red beans.

Bean Storage. Control beans were stored at room temperature conditions (21 °C and 50% relative humidity) (*20*) for 5 months. To develop the HTC defect, beans were stored for 5 months at 30 °C and 65% relative humidity. Three plastic buckets (approximately 30 L) containing a supersaturated salt solution were used to create the environment with the latter conditions. Ammonium chloride (1500 g) was mixed with 900 mL of deionized−distilled water (DDW) and placed in the bottom of each plastic bucket. A plastic base with holes was placed about 8 cm above the level of the salt solution to prevent the beans from coming into contact with the salt solution. Six bags (woven cloth bags with drawstring, 200 g/bag) of each bean cultivar were randomly placed inside the buckets (two bags/bean cultivar/bucket). Finally, the buckets were tightly sealed with lids, laboratory film (Parafilm M, Menasha, WI), and grease (Silicone, High Vacuum Stop-Cock Lubricant, Dow Corning 976V, Midland, MI) to maintain the internal required conditions and finally placed in an incubation chamber at 30 °C.

Sample Preparation. The bean seeds were randomly divided into 36 bags of 200 g each (12 bags per cultivar). Fifty grams of whole bean seeds was ground to a 40-mesh particle size using a Cyclotec1093 sample mill (Tecator, Hoganas, Sweden) and were subsequently ground again using a Retsch mixer mill model MM2 (Brinkmann Instruments, Inc., Des Plaines, IL) to achieve a 60-mesh particle size. The fine powder was stored at −80 °C prior to phenolic acid extraction.

Cooking Time. A cooking test was done to ensure that beans developed the HTC defect. The method was performed as outlined by Durkee (*21*) and Hentges et al. (*17*) with minor modifications. Before cooking, seed samples (20 g) were soaked for 16 h in 60 mL of DDW at 21 °C. The extent of the HTC defect was measured using a Mattson-type bean cooker (*22*). Twenty-five beans were placed in the apparatus underneath probes weighing 105 ± 1 g each, and the apparatus was placed in boiling water (100 °C). Beans were cooked until such softness that the tip of each probe passed through the bean. The time at which each probe dropped was recorded. Fifty percent cooking times were calculated as the average between the time at which the 12th and 13th probes passed through the beans. The analysis was done in triplicate for both control and HTC beans.

Extraction of Phenolic Acids from Beans. The method used for the extraction of phenolic acids was adapted from that of Hernanz et al. (*23*) with modifications. One gram of each ground bean sample was weighed into a 50 mL polystyrene centrifuge tube and extracted with 20 mL of a mixture of 50% methanol, 48% water, and 2% 6 M HCl to release the free phenolic acids (FPA). Samples were blanketed with nitrogen gas and placed in an ultrasonic bath for 1 h. Samples then were centrifuged at 9000*g* at 21 °C for 10 min, and supernatants were collected for analysis. Bean residues from the first extraction were mixed with 10 mL of 2 M NaOH to release the alkaline hydrolyzable phenolic acids (BHPA). Samples were blanketed with nitrogen gas and placed in an ultrasonic bath for 2 h. Next, 10 mL of a combination of 50% methanol, 48% water, and 2% acetic acid was added to the samples and again placed in an ultrasonic bath for 15 min. Then the samples were centrifuged at 9000*g* at 21 °C for 10 min, and the supernatant was retained for analysis. Finally, 10 mL of 6 M HCl was added to the residue from the alkaline hydrolysis to release the acid hydrolyzable phenolic acids (AHPA). Samples were blanketed with nitrogen gas and placed in an ultrasonic bath for 1 h. Next, 10 mL of 50% methanol, 48% water, and 2% acetic acid was added to the samples and placed again in an ultrasonic bath for 15 min. Then the samples were centrifuged at 9000*g* at 21 °C for 10 min, and the supernatant was retained for analysis. All of the extracts were filtered first with a 0.45 μm polytetrafluoroethylene (PTFE) filter and then with a 0.2 μm nylon filter (both Fisher brand) and stored at −80 °C for 1 week before the analyses were performed.

Determination of Total Phenolic Content. A modified Folin−Ciocalteu assay as described by Escarpa and Gonzalez (*24*) was used to measure the total content of phenolic acids in the extracts. Previous studies have shown that ferulic acid is one of the most abundant simple phenolic acids in normal and HTC beans (*12, 13*). Therefore, a standard curve of FA with concentrations ranging from 10 to 300 mg/L in a solution of 50% HPLC grade methanol, 48% water, and 2% acetic acid was plotted to determine the concentration of phenolic acids in the samples.

Similar amounts (54 μL) of extract or standard and Folin−Ciocaletau reagent were mixed and allowed to react for 5 min at 21 °C. Subsequently, 1 mL of a solution of 0.7 M Na_2CO_3 was added and allowed to react for 1 h at 21 °C in the dark, and the absorbance of the mixture was measured at 750 nm in a spectrophotometer (Beckman DU640, Fullerton, CA). Sample blanks were done in each assay. The concentration of phenolic acids in the extracts was expressed as milligrams of ferulic acid equivalents per gram of dry beans.

Determination of Antioxidant Capacity of Bean Extracts. The antioxidant activity of the three extracts (FPA, BHPA, and AHPA) of beans was determined by measuring the ability of the compounds in each extract to scavenge the radical $ABTS^+$, expressed as Trolox equivalent antioxidant capacity (TEAC), according to the method of Re et al. (*25*) with modifications. The $ABTS^+$ radical cation was prepared in an amber vial by mixing equal amounts of ABTS (0.014 M in DDW) and potassium persulfate (0.0049 M in DDW) and then reacting in the absence of light at 21 °C for 12 h. Before the analysis, the $ABTS^+$ was diluted to a ratio of 1:99 with DDW and adjusted to an absorbance of 0.600 ± 0.010 at 734 nm in a spectrophotometer (Beckman DU640).

The extracts were diluted to different ratios to obtain an estimated uniform concentration of phenolic acids (using the results from Folin−Ciocalteau assay) among all samples. Subsequently, 33 μL of extract or standard was mixed with 1 mL of the $ABTS^+$ solution. This mixture was allowed to react in the absence of light at 21 °C for 15 min, and then the absorbance was read at 734 nm. Sample blanks (SB, 33 μL of sample plus 1 mL of DDW), $ABTS^+$ blank for the standard curve (ABC, 33 μL of methanol plus $ABTS^+$ diluted), and $ABTS^+$ blank for samples (ABS, 33 μL of extract solvent plus $ABTS^+$ diluted) were completed for each assay.

A standard curve was created using Trolox (*25*) with concentrations ranging from 6 to 48 mg/L in HPLC grade methanol. The change in absorbance (absorbance of ABC − absorbance of $ABTS^+$ plus Trolox solutions) and the concentration of each Trolox solution were plotted on the standard curve. The change in absorbance for each extract was calculated using the following equation:

$$\Delta\text{Abs sample} = \text{Abs ABS} - [(\text{Abs } ABTS^+ + \text{sample}) - \text{Abs SB}]$$

The equivalent concentration of Trolox compared to the change in absorbance for each extract was calculated using the equation from the standard curve. Finally, the TEAC value for each extract was calculated using the following equation:

$$\text{TEAC} = \frac{\text{equivalent concn of Trolox (mg/L) for each extract}}{\text{extract concn (mg/L) (results from Folin−Ciocalteu assay)}}$$

The TEAC value represents the relative antioxidant capacity that 1 unit of weight of the phenolic acids present in the extracts has compared with 1 unit of weight of Trolox.

Preparation of Standards for HPLC Analysis. Stock solutions (20 ppm) were prepared by dissolving individual standards (gallic, protocatechuic, chlorogenic, caffeic, syringic, *p*-coumaric, ferulic, and sinapic acids) in a mixture of HPLC grade methanol, water, and acetic acid (50:48:2 v/v/v). The eight standard stock solutions were mixed in equal amounts to make a standard mixture and diluted to get concentrations from 0.01 to 1.00 ppm for each acid.

High-Performance Liquid Chromatography Analysis. An HPLC system with an electrochemical array detector was used to identify the phenolic acids. The system was configured with two solvent delivery pumps, an autosampler, a column (Waters, Nova-Pak C18 column, 150 × 3.9 mm, particle size = 4 μm), a 75 μL sample loop, and a 12-channel CoulArray detector.

The two mobile phases consisted of (A) 1 M ammonium acetate, pH 4.5, acetic acid, and water (2:2:96, v/v/v) and (B) 1 M ammonium acetate, pH 4.5, and acetonitrile (2:98, v/v). All of the solvents used were of HPLC grade. The flow rate was 0.9 mL/min. The mobile phase composition began with 100% A, which was maintained for 1 min, followed by a linear increase to 100% B in 52 min, held at 100% B for 5 min, and later decreased to 45% B over 5 min and to 5% B over 2 min; finally it was returned to initial conditions in 2 min. The injection volume was 20 μL for the FPA extracts and 40 μL for the AHPA and BHPA extracts. Eight channels were used for the analysis, and the cell potentials were set from 100 to 800 mV with an increase of 100 mV between consecutive cells.

Statistical Analysis. Three-digit random numbers were assigned to each bag of beans to minimize sample bias during the experiment. A two-factor full-factorial design was used. The two factors investigated were bean cultivar [with three levels: T-39 (black), Lebaron (red), and Seahawk (white)] and treatment (with two levels: control and HTC beans). Experiments for the determination of 50% cooking time were carried out in triplicate. Six replicates with two determinations for each replicate were used for the determination of the total content of phenolic acids and the HPLC analysis. Three determinations for each replicate were used to determine the antioxidant capacity. Statistical analysis of the data was done using SAS (SAS Institute Inc., Cary, NC). Some of the data sets were transformed using Box−Cox transformation to achieve a linear model. Statistical analyses were conducted using PROC GLM and a Bonferroni multiple means comparison test. All dependent variables as affected by treatment, bean cultivar, and treatment−bean cultivar interaction were investigated using analysis of variance (ANOVA). Results were considered to be significant if $P < 0.05$. The error bars drawn on the graphs represent the standard deviation obtained after Box−Cox transformation of the data.

RESULTS AND DISCUSSION

Cooking Time. The major characteristic of HTC beans is prolonged cooking time. The 50% cooking time for bean seeds stored at room temperature conditions and at high temperature and relative humidity are shown in **Table 1**. The results indicated a significant increase ($P < 0.05$) in the cooking time of HTC beans compared to the controls for all three bean cultivars. The cooking times of the HTC beans were about 3, 5, and 6 times higher than those of the control beans for the T-39 (black), Lebaron (red), and Seahawk (white) cultivars, respectively. Consistent with our data, previous research reported increases from 1.5 to 10 times in the 50% cooking time for beans that were stored under conditions of high humidity and temperature (*17, 18*). The 50% cooking times for the red and white control beans were not significantly different, nor were the 50% cooking times for the red and white HTC beans ($P < 0.05$).

Table 1. Average 50% Cooking Times for Control and Hard-to-Cook (HTC) Red, Black, and White Beans Using a Mattson-Type Bean Cooker

	av 50% cooking time[a] (min)	
bean cultivar	control beans[b]	HTC beans[c]
T-39 (black)	18.22 ± 1.85aA	60.75 ± 6.92bA
Lebaron (red)	16.77 ± 1.62aAB	87.93 ± 6.35 bB
Seahawk (white)	14.48 ± 0.71aB	93.12 ± 6.85bB

[a] Values are expressed as the average of triplicate analyses ± standard deviation. Values in the same column with different capital letters are significantly different according to a Bonferroni multiple-comparison test ($P < 0.05$). Lower case letters within the same bean cultivar indicate statistical significance between the values listed in the control and HTC columns according to a Bonferroni multiple-comparison test ($P < 0.05$). [b] Control beans were stored at 21 °C and 50% relative humidity for 5 months. [c] HTC beans were stored at 29 °C and 65% relative humidity for 5 months.

The HTC white beans did not have a significantly different cooking time when compared to the red beans but did when compared with the black cultivar. These results are in agreement with those of Muller (*26*), who showed that HTC beans with a darker seed coat had a lower hardness (shorter cooking time) than beans with a clear seed coat. Although the dark beans have a higher content of lignin, which is primarily deposited in the middle lamella of the cell wall and acts as a binding substance among the cells, they may possess a thinner palisade layer and have much lower α-cellulose content in the seed coats than colorless beans (*26*). This may indicate that cell walls of colored beans are less tough.

Total Phenolic Content. The T-39 (black) cultivar had the highest concentration of phenolic acids for both control and HTC beans compared to the Lebaron (red) and Seahawk (white) cultivars (**Figure 1**). The content of phenolic acids in the FPA fraction obtained in this study (1.01−5.109 mg of ferulic acid equiv/g of bean) partially agreed with the results by Heimler (*11*), who reported a range of content of free phenolic acids from 1.17 to 4.40 mg of gallic acid equiv/g of beans for several bean cultivars. However, the cultivars and seed coat color were not indicated. Also, Bressani et al. (*27*) reported concentration ranges for total phenolic acids of 2.48−18.86, 1.50−3.26, and 1.87−10.06 mg of catechin equiv/g for black, white, and red beans, respectively. In contrast, the results of the current study did not agree with the results of Ganthavorn et al. and Bressani (*28, 29*), who reported concentrations for free phenolic acids of 5.61 and 14.7, 3.87 and 9, and 2.35 and 3.6 mg of chlorogenic acid equiv/g bean for two different cultivars of red, black, and white beans. The discrepancy in results might be attributed to the previous studies employing different methods for the extraction and estimation of phenolic acids (i.e., time of extraction, different ratios of methanol/water for the extraction), using different bean cultivars (i.e., genetic differences), using different phenolic acid standards to report the concentration, and the beans having different growing and storage conditions (i.e., level of minerals in fields, different temperatures and relative humidities during storage).

A significant difference ($P < 0.05$) between the content of FPA in control and HTC beans was observed for the black and red cultivars (**Figure 1A**). There was no significant difference between control and HTC beans for all three cultivars ($P < 0.05$) for the content of alkaline hydrolyzable phenolic acids (BHPA). However, the white cultivar had a significant difference ($P < 0.05$) between control and HTC beans (**Figure 1B**). Finally, black and red beans were not statistically different ($P < 0.05$) in the content of acid hydrolyzable phenolic acids (AHPA) (expressed as mg of ferulic acid equiv/g bean) for

Table 2. Phenolic Acid Content in Control and Hard-to-Cook (HTC) Beans in Free Phenolic Acids (FPA) Fraction by High-Performance Liquid Chromatography with Electrochemical Detection

		mean concn of acids[a] (μg/g of beans)													
		gallic		protocatechuic		caffeic		syringic		*p*-coumaric		ferulic		sinapic	
bean cultivar	treatment	mean (SD)[b]	*n*	mean (SD)	*n*	mean (SD)	*n*	mean (SD)	*n*	mean (SD)	*n*	mean (SD)	*n*	mean (SD)	*n*
T-39 (black)	control	2.23a (0.13)	6	9.09a (2.75)	6	0.97a (0.26)	6	nd		1.16a (0.28)	6	3.85a (1.06)	6	0.55a (0.05)	6
	HTC	3.87b (0.36)	6	4.13b (0.72)	6	1.14a (0.29)	4	3.03 (0.11)	5	2.06b (0.34)	5	15.25b (2.02)	5	2.53b (0.63)	5
Lebaron (red)	control	nd[c]		1.85a (0.37)	6	nd		nd		1.30a (0.16)	6	1.44a (0.08)	6	0.64a (0.02)	6
	HTC	nd		1.74a (0.23)	6	0.61 (0.03)	6	1.19 (0.12)	6	2.01b (0.41)	6	12.53b (0.67)	6	4.25b (0.21)	6
Seahawk (white)	control	nd		0.14a (0.03)	6	nd		nd		0.82a (0.39)	6	0.77a (0.09)	6	0.49a (0.03)	6
	HTC	nd		0.17a (0.01)	6	0.45 (0.02)	6	nd		1.41b (0.06)	6	10.27b (0.80)	6	3.37b (0.34)	6
retention time (min)	control	3.88 (0.05)		6.63 (0.20)		12.90 (0.07)		nd		17.86 (0.34)		21.57 (0.34)		22.42 (0.34)	
	HTC	3.95 (0.06)		6.74 (0.26)		12.85 (0.38)		13.67 (0.54)		18.56 (0.50)		21.52 (0.48)		22.42 (0.53)	

[a] Means in a column within a bean cultivar followed by different letters are significantly different ($P < 0.05$). [b] Standard deviation given in parentheses. [c] nd, not detectable.

both control and HTC beans. Only the black cultivar showed a statistical difference ($P < 0.05$) in AHPA between control and HTC beans (**Figure 1C**).

Storage conditions that caused the HTC phenomenon did not have the same effect on all phenolic acid fractions in the three bean cultivars. There was a considerable loss of FPA in the HTC beans of approximately 40% in black beans (**Figure 1A**). This result was similar to a study conducted by Stanley et al. (*30*), who reported a decrease of 41% in extractable phenols in HTC beans. It was hypothesized that the total phenols became less extractable during storage at tropical conditions, because they become involved in a cross-linking reaction that inhibits water imbibition, which restricts water uptake and reduces cell separation. Phenolic compounds are known to form complexes with proteins and carbohydrates (*31, 32*).

The storage that caused the HTC phenomenon decreased the content of FPA in black and red beans (**Figure 1A**). These two cultivars may have higher contents of tannins than white cultivars. According to the literature, most phenolic compounds present in the FPA fraction are considered to be tannins (*19, 33*−*35*). Studies showed that condensed tannins decreased over storage time in common black beans (*35*) or milled bean flour (*32*), along with a negative correlation between tannin content and the development of hardness. This decrease in tannin content may be due to polymerization of these existing high molecular weight polyphenolic compounds to insoluble polymers such as lignins (*32, 34*) or their migration into the cotyledon (*35*).

During the storage of beans under conditions of high humidity and temperature, there is an increase in liberation or synthesis of free phenolic acids, such as ferulic acid in the cotyledon, providing phenolic compounds to cross-link with proteins in the middle lamella (*13*). This protein−phenol interaction may increase protein hydrophobicity, resulting in a subsequent decrease in seed hydration during cooking. This interaction could restrict cell separation during cooking (*32*), consequently exacerbating the HTC defect. In addition, it is known that tannins are high molecular weight compounds containing sufficient phenolic hydroxyl groups to permit the formation of stable cross-links with proteins by peroxidase and/or free radicals from membrane breakdown that would result in reduced extractability (*30*). Furthermore, the loss of tannins may be attributable to the presence of polyphenol oxidase and to enzymatic hydrolysis (*18, 19*).

On the basis of this study, there was not a statistical difference in the concentration of FPA between control and HTC beans for the white cultivar. The literature has reported a low or nondetectable level of tannins in white cultivars (*2, 33*−*35*). The increase in cooking time has been related to a decrease in tannin concentration; however, the white beans showed an increase in cooking time, meaning that the loss in tannins in the HTC black and red beans could be only one factor related to the HTC phenomenon.

The control white bean was the only bean cultivar that showed a higher concentration ($P < 0.05$) of BHPA for HTC beans than for control beans (**Figure 1B**). This finding was similar to the results of Srisuma et al. (*13*), who observed that the seed coat of HTC navy (white) beans had an increase in phenolic acid content (both free and bound forms), but there was a decrease within the cotyledons. The reason there was not a significant difference in the concentration of phenolic acids in the BHPA fractions in both black and red cultivars could be because of the short time of acid and alkaline hydrolysis (1 and 2 h, respectively) used in this study, which could have reduced the amount of phenolic acids released. Previous studies have used extended times of hydrolysis such as 16 h (*13, 23*). However, in this study, short times for alkaline and acid hydrolysis were used to reduce the negative effect that extreme pH may have on the antioxidant capacity of the phenolic acids extracted during hydrolysis. In addition, a prolonged time of hydrolysis can lead to the formation of complexes between phenolic compounds and proteins or carbohydrates (*31*), decreasing the amount of extractable phenolic acids. A probable consequence would be an underestimation of the true phenolic acid content.

Antioxidant Capacity. For this study, beans of three different seed coat colors were selected because previous studies have shown that the antioxidant activity of fruits and vegetables is positively correlated with the content of phenolic compounds in the seed coat. Therefore, it was assumed that the legumes with the highest phenolic content were dark cultivars, such as black and red beans (*3, 9*).

The black bean cultivar exhibited the highest antioxidant activity for FPA and AHPA fractions in both control and HTC beans (**Figure 2A**,**C**). For the BHPA fraction, there was no statistical difference ($P < 0.05$) between the black and red bean cultivars for both control and HTC beans (**Figure 2B**). The white cultivar showed the lowest antioxidant capacity of the three bean cultivars for all phenolic acid fractions (**Figure 2**). Overall, the storage that caused the HTC phenomenon did not affect the antioxidant capacity for the BHPA fraction in all three cultivars.

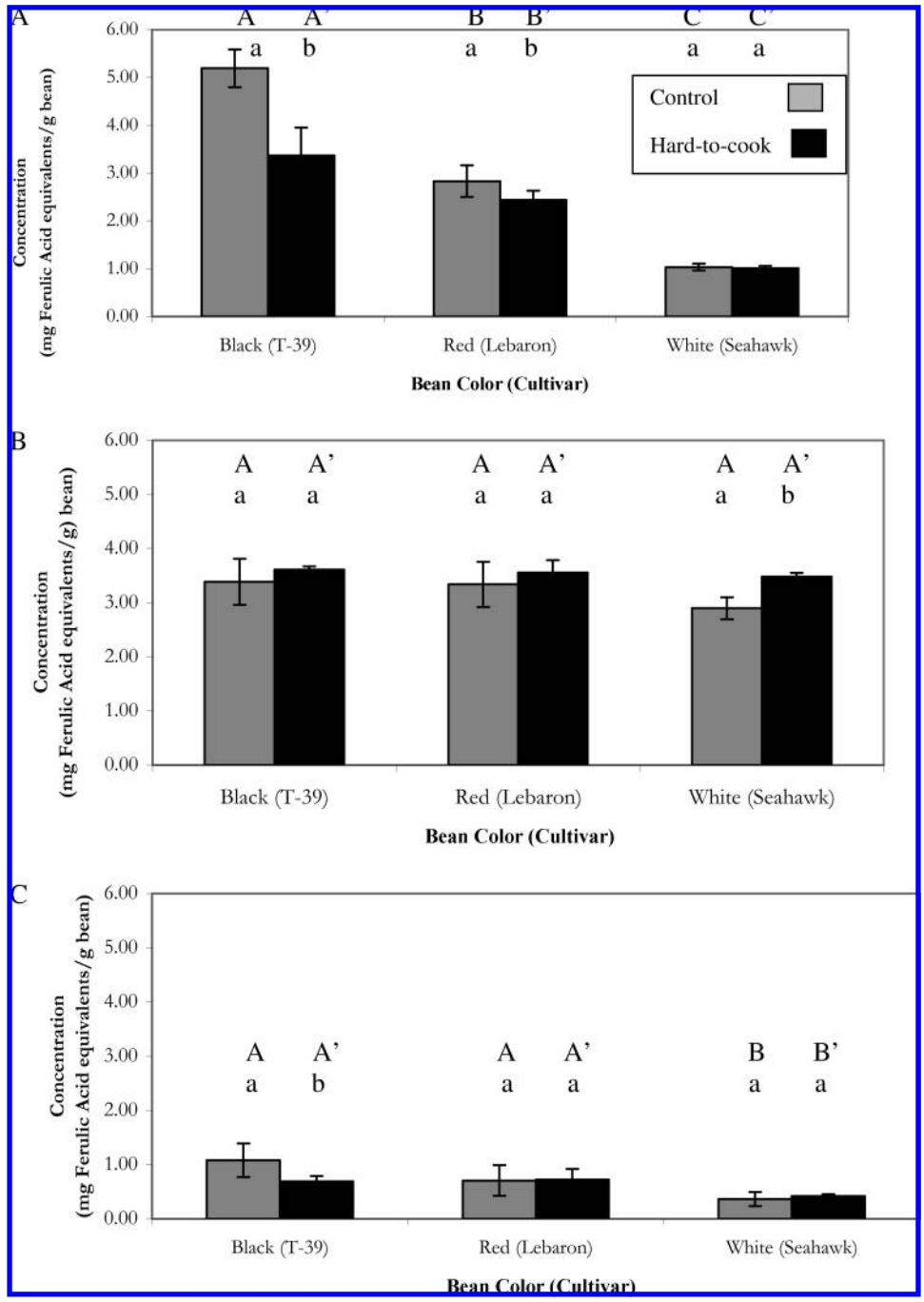

Figure 1. Mean concentrations of phenolic acids (mg of ferulic acid equivalents/g of bean) in two treatments, control and hard-to-cook (HTC) beans among three cultivars: (**A**) free phenolic acids (FPA); (**B**) alkaline hydrolyzable phenolic acids (BHPA); (**C**) acid hydrolyzable phenolic acids (AHPA). Different capital letters above the bars indicate significant differences ($P < 0.05$) among the three bean cultivars within each treatment. Different lower case letters above the bars indicate significant differences ($P < 0.05$) between control and HTC beans within each bean cultivar. All of the results are according to a Bonferroni multiple-comparison test.

The FPA and AHPA fractions for both black and white cultivars were the most affected by the storage.

The FPA fractions for both control and HTC black beans in this study had the highest antioxidant activity among all three cultivars. This finding differed from that of Benninger et al. (*2*), who determined that free phenolic compounds in red bean cultivars had higher antioxidant activity than those in black bean cultivars. However, only seed coats were analyzed and not the whole bean seeds. Therefore, other phenolic compounds present in the cotyledons could have affected the antioxidant capacity of the whole beans. Kim et al. (*8*) stated that the antioxidant activity of plant extracts indicates only the total antioxidant capacity of the mixture of compounds; however, the role of individual phenolic acids or other compounds and their contribution to the total antioxidant activity cannot be deciphered from the mixture.

In most cases, storage reduced the antioxidant capacity of the compounds present in the FPA fraction for the black and white bean cultivars, as well as for the compounds in the AHPA fraction for all three bean cultivars. The literature has reported an increase in simple phenolic acids in HTC beans as a stress response when the beans were stored under adverse conditions (*13*). However, these phenolic acids, which could provide antioxidant activity, contain hydroxyl groups that allow the

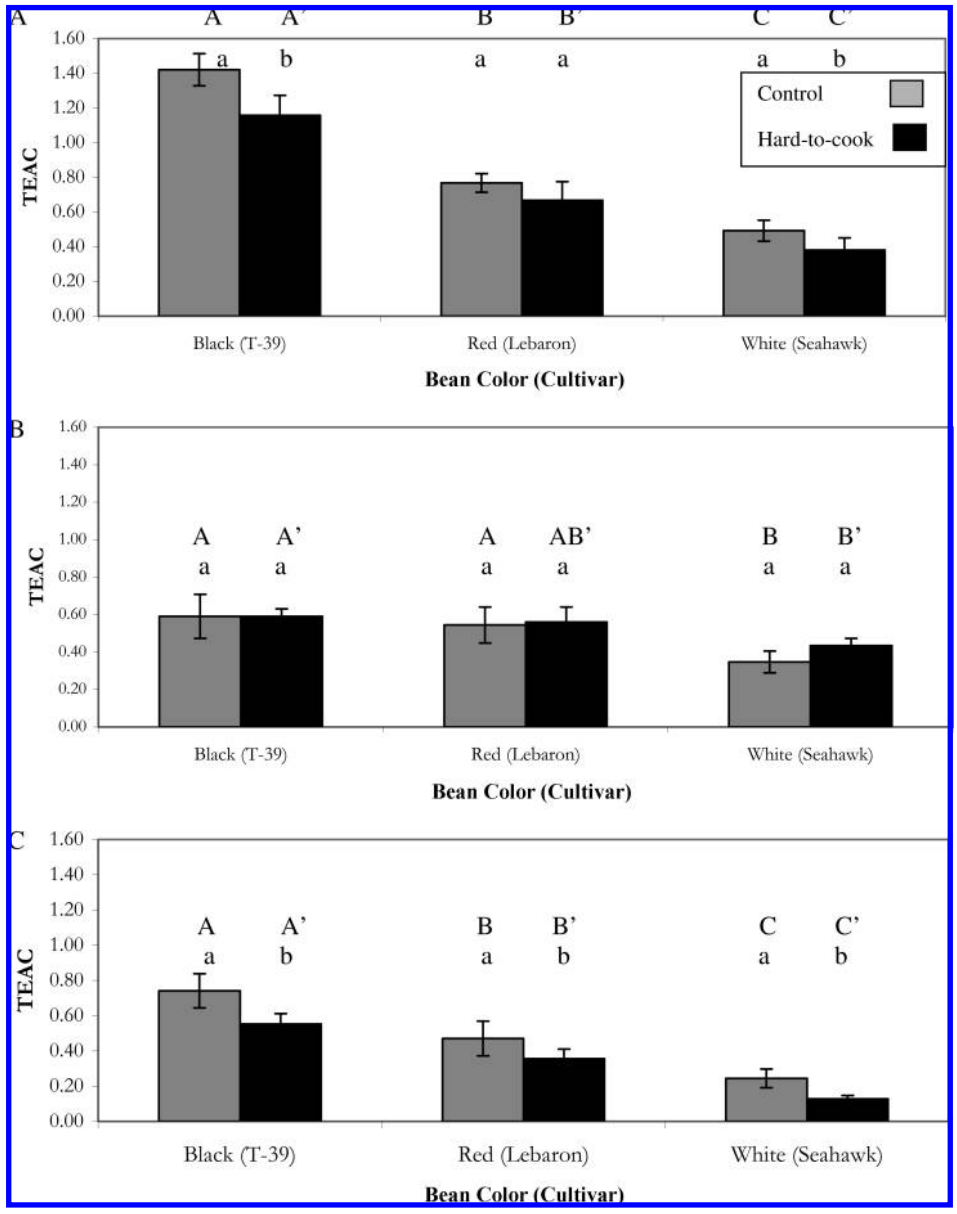

Figure 2. Mean Trolox equivalent antioxidant capacity (TEAC) in two treatments, control and hard-to-cook (HTC) beans among three cultivars: (**A**) free phenolic acids (FPA); (**B**) alkaline hydrolyzable phenolic acids (BHPA); (**C**) acid hydrolyzable phenolic acids (AHPA). Different capital letters above the bars indicate significant differences ($P < 0.05$) among the three bean cultivars within each treatment. Different lower case letters above the bars indicate significant differences ($P < 0.05$) between control and HTC beans within each bean cultivar. All of the results are according to a Bonferroni multiple-comparison test.

formation of stable cross-links with proteins. This cross-linkage can reduce the antioxidant potential of the phenolic acids, because the potential is linked in part to the number of available hydroxyl groups of the phenolic acids. Stanley (*35*) reported a decrease in the total content of free phenolic compounds (specifically tannins) for HTC black beans. A significant amount of the antioxidant activity found in methanol extracts (FPA) of beans may be due to the condensed tannins present (*2*). In other words, a decrease in tannins could represent a decrease in antioxidant activity for black beans.

On the basis of this study, the BHPA fractions may have higher antioxidant activity than the AHPA fractions for almost all of the cultivars. This finding is consistent with the results of a study by Kim et al. (*8*), who observed that the alkaline hydrolyzable fractions of different wheat brans had greater antioxidant activities than the acid hydrolyzable fraction. The BHPA fractions could have higher antioxidant activity than the AHPA fractions because the concentration of simple phenolic acids, such as protocatechuic, caffeic, syringic, *p*-coumaric, ferulic, and sinapic acids (**Tables 3** and **4**), detected by the HPLC analysis, was higher in the former fraction than in the latter.

The Pearson correlation coefficients between the content of phenolic acids and the antioxidant activity for all of the data, and for separate control and HTC beans, are shown in **Table 5**. In general, the content of phenolic acids and the antioxidant capacity of all the samples were moderately correlated ($r = 0.58$, $P < 0.0001$). These results agreed with the results reported by Adom et al. (*36*), who noted a strong correlation ($r = 0.81$, $P = 0.002$) between the total phenolic content of wheat extracts and antioxidant activity. In this study, the content of FPA and antioxidant activity of the FPA fraction seemed to be more highly correlated than the concentration and antioxidant activity of the AHPA fraction. The concentration and antioxidant activity of the BHPA fraction were not significantly correlated. These results agreed with the results reported by Cardador-Martinez

Table 3. Phenolic Acid Content in Control and Hard-to-Cook (HTC) Beans in Alkaline Hydrolyzable Phenolic Acids (BHPA) Fraction by High-Performance Liquid Chromatography with Electrochemical Detection

		mean concn of acids[a] (μg/g of beans)													
		gallic		protocatechuic		caffeic		syringic		*p*-coumaric		ferulic		sinapic	
bean cultivar	treatment	mean (SD)[b]	*n*	mean (SD)	*n*	mean (SD)	*n*	mean (SD)	*n*	mean (SD)	*n*	mean (SD)	*n*	mean (SD)	*n*
T-39 (black)	control	nd[c]		1.45a (0.14)	6	0.64a (0.20)	6	4.34b (0.64)	6	1.06a (0.36)	6	7.87a (2.56)	5	3.43a (0.57)	6
	HTC	nd		0.72b (0.04)	6	0.60a (0.03)	4	5.95a (0.67)	6	0.91a (0.52)	6	6.84a (1.99)	5	3.39a (1.33)	6
Lebaron (red)	control	nd		4.04a (0.85)	6	nd		nd		0.80a (0.12)	4	7.65a (1.28)	6	3.51a (0.48)	6
	HTC	nd		1.33b (0.15)	6	0.60 (0.14)	5	nd		0.78a (0.51)	6	7.60a (1.56)	6	4.76a (0.87)	5
Seahawk (white)	control	nd		nd		nd		nd		0.75 (0.14)	6	6.69a (1.97)	5	3.32a (0.77)	6
	HTC	nd		nd		nd		nd		nd		3.75b (1.40)	5	1.80b (0.74)	6
retention time (min)	control			6.93 (0.12)		12.98 (0.11)		13.88 (0.03)		18.15 (0.40)		21.66 (0.31)		22.56 (0.31)	
	HTC			6.79 (0.10)		12.95 (0.09)		13.74 (0.12)		18.38 (0.34)		21.80 (0.29)		22.85 (0.29)	

[a] Means in a column within a bean cultivar followed by different letters are significantly different ($P < 0.05$). [b] Standard deviation given in parentheses. [c] nd, not detectable.

Table 4. Phenolic Acid Content in Control and Hard-to-Cook (HTC) Beans in Acid Hydrolyzable Phenolic Acids (AHPA) Fraction by High-Performance Liquid Chromatography with Electrochemical Detection

		mean concn of acids[a] (μg/g of beans)													
		gallic		protocatechuic		caffeic		syringic		*p*-coumaric		ferulic		sinapic	
bean cultivar	treatment	mean (SD)[b]	*n*	mean (SD)	*n*	mean (SD)	*n*	mean (SD)	*n*	mean (SD)	*n*	mean (SD)	n	mean (SD)	*n*
T-39 (black)	control	2.44a (0.73)	6	0.63a (0.20)	6	0.48a (0.15)	6	0.77a (0.21)	6	0.48a (0.05)	5	1.91a (0.43)	5	0.54a (0.12)	5
	HTC	2.25a (0.63)	6	1.01a (0.13)	6	0.19b (0.04)	6	0.91a (0.16)	6	0.41a (0.06)	6	1.76a (0.53)	6	0.71a (0.19)	6
Lebaron (red)	control	nd[c]		1.38a (0.37)	6	0.26a (0.06)	6	nd		0.42a (0.18)	6	1.92a (0.37)	5	0.65a (0.18)	4
	HTC	nd		1.72a (0.27)	6	0.21a (0.05)	6	nd		0.40a (0.07)	6	2.09a (0.46)	6	1.67b (0.57)	6
Seahawk (white)	control	nd		nd		0.21a (0.10)	5	nd		0.44a (0.16)	6	2.13a (0.39)	5	0.64a (0.13)	5
	HTC	nd		nd		0.15a (0.02)	5	nd		0.37a (0.05)	6	1.35b (0.42)	6	0.45a (0.10)	6
retention time (min)	control	3.89 (0.02)		6.67 (0.04)		12.56 (0.08)		13.44 (0.09)		17.72 (0.10)		21.08 (0.10)		22.00 (0.09)	
	HTC	3.91 (0.06)		6.44 (0.02)		12.47 (0.05)		13.27 (0.03)		17.52 (0.23)		21.04 (0.07)		22.09 (0.09)	

[a] Means in a column within a bean cultivar followed by different letters are significantly different ($P < 0.05$). [b] Standard deviation given in parentheses. [c] nd, not detectable.

et al. (*10*), who noted a correlation coefficient >0.91 ($P < 0.0001$) between the phenolic content of methanolic extracts (FPA) of beans and antioxidant activity. A poor correlation between the content of bound phenolics and the antioxidant activity was reported (*10*). The authors suggested that the poor correlation between bound phenolics and the antioxidant activity was because these phenolic acids were covalently bound and this factor could affect the antioxidant potential of the acids (*10*).

Identification of Individual Phenolic Acids. The contents of simple phenolic acids in the FPA, BHPA, and AHPA fractions for both control and HTC beans are shown on **Tables 2**, **3**, and **4**, respectively. Protocatechuic, *p*-coumaric, ferulic, and sinapic acids were those most commonly detected in all three fractions and in most of the bean cultivars. These results concurred with previous studies, in which *p*-coumaric, sinapic, and ferulic acids were identified in bean extracts, with the latter acid being the most abundant (*5, 6, 12, 13, 37*). However, the concentrations reported in these studies were higher than those reported in the current study.

For control beans, the concentration of ferulic acid and sinapic acid seemed to be higher in the BHPA fraction than in the FPA fraction for all bean cultivars. Luthria et al. (*6*) reported insignificant amounts of simple phenolic acids in beans in the free fraction; however, alkaline hydrolysis of bean extracts provided the majority of the phenolic acids. In contrast, for the HTC beans, the concentration of ferulic and *p*-coumaric acids seemed to be higher in the FPA fraction than the BHPA fraction. In general, for all bean cultivars and control and HTC beans, the AHPA fraction had the lowest concentration of *p*-coumaric, ferulic, and sinapic acids. These results were not consistent with a study by Luthria et al. (*6*), who reported that the sequential acid hydrolysis of bean residues from a previous alkaline hydrolysis did not yield significant amounts of free phenolic acids.

The major differences in the concentrations of simple acids were for *p*-coumaric, sinapic, and ferulic between HTC and control beans in the FPA fraction for all three bean cultivars (**Table 2**). For the BHPA and AHPA fractions, no major differences were detected between control and HTC for black and red beans (**Tables 3** and **4**). These results partially agreed with a study by Srisuma et al. (*13*), who observed an increase in the concentration of ferulic, sinapic, and *p*-coumaric acids in navy (white) HTC beans in both free and bound phenolic acid fractions. In the current study, the white cultivar only showed an increase in the concentration of those acids in the FPA fraction and a decrease in the BHPA fraction. Furthermore, the concentrations of simple phenolic acids reported by Srisuma et al. (*13*) were higher than those obtained in the current study. These differences could be due to the fact that Srisuma et al. (*13*) used a different phenolic acid extraction procedure that included the use of enzymes to release bound phenolic acids, in addition to longer times of hydrolysis. The increase in free phenolic acid content in HTC beans could be a false germination

Table 5. Pearson Correlation Coefficients between Trolox Equivalent Antioxidant Capacity and Content of Phenolic Acids for Control and Hard-to-Cook (HTC) Beans

Treatment	data		Pearson correlation coefficient (r)	P value[a]
Control and HTC beans	all data (control and HTC beans)		0.58	<0.0001
	by bean cultivar	black (T-39)	0.51	0.0013
		red (Lebaron)	0.40	0.0161
		white (Seahawk)	0.37	0.0245
	by phenolic acid fraction	free phenolic acids (FPA)	0.80	<0.0001
		alkaline hydrolyzable phenolic acids (BHPA)	0.13	0.4391
		acid hydrolyzable phenolic acids (AHPA)	0.55	0.0005
	by treatment	control beans (normal)	0.62	<0.0001
		HTC beans	0.52	<0.0001
Control beans		control beans	0.62	<0.0001
	by bean cultivar	black (T-39)	0.57	0.0133
		red (Lebaron)	0.25	0.3113
		white (Seahawk)	−0.04	0.8886
	by phenolic acid fraction	free phenolic acids (FPA)	0.88	<0.0001
		alkaline hydrolyzable phenolic acids (BHPA)	0.06	0.8112
		acid hydrolyzable phenolic acids (AHPA)	0.47	0.0480
		HTC beans	0.52	<0.0001
HTC beans	by bean cultivar	black (T-39)	0.42	0.0798
		red (Lebaron)	0.57	0.0141
		white (Seahawk)	0.72	0.0008
	by phenolic acid fraction	free phenolic acids (FPA)	0.89	<0.0001
		alkaline hydrolyzable phenolic acids (BHPA)	0.26	0.2983
		acid hydrolyzable phenolic acids (AHPA)	0.64	0.0039

[a] $P < 0.05$ is statistically significant.

response under high temperature and relative humidity conditions (*32*). Under these conditions, storage proteins could be mobilized and degraded by the action of proteases to simple phenolic acids. Also, an increase in ferulic acid concentration could represent cell wall breakdown.

The concentration of total phenolic acids obtained from the Folin−Ciocalteu assay was much higher than the concentration of the individual phenolic acids obtained through the HPLC analysis. This observation agreed with a study by Escarpa et al. (*24*), who reported that the Folin−Ciocalteu assay overestimated the content of phenolic content in different foods when compared to the HPLC analysis. Although this spectrophotometric method lacks selectivity, and there are compounds that could interfere such as sugars, proteins, and ascorbic acid, this method is still widely employed in analytical laboratories for the assay of phenolic content. In addition, the extracts analyzed in this study may contain other phenolic acids that were detected by the Folin−Ciocalteu assay but were not quantified through HPLC analysis.

On the basis of this study, there was a decrease in the total content of phenolic acids and antioxidant activity for the HTC beans for some of the bean cultivars and phenolic fractions. In contrast, the results of the HPLC analysis showed an increase or minor changes in the concentration of simple phenolic acids for the FPA fraction and the BHPA and AHPA fractions, respectively. However, the concentrations of the acids detected by the HPLC analysis were very low (μg/g bean) compared to the total content of phenolic acid (mg/g). This disagreement could mean that there are other phenolic compounds in beans which could have more potent antioxidant activity and were present in higher concentrations than the simple phenolic acids identified through HPLC analysis.

In conclusion, the storage that caused the HTC phenomenon did not have the same effect on total concentration and antioxidant capacity of all three fractions of phenolic acids in all three bean cultivars. The total concentration and the antioxidant capacity of free and acid hydrolyzable phenolic acids in black beans were most affected by the storage. The concentration of *p*-coumaric, ferulic, and sinapic acid in the free phenolic acids fraction for all three bean cultivars increased in HTC beans. In general, the total content of phenolic acids was moderately correlated to the antioxidant activity, and this correlation was much higher between the total content of free phenolic acids and the antioxidant activity. The HTC phenomenon did not affect the total concentration and the antioxidant capacity in the same way. Further work is needed to identify changes in other potential antioxidants present in the extracts.

Finally, on the basis of the results of this study, the black beans (T-39 cultivar) could represent the best source of phenolic acids with the highest antioxidant capacity among the cultivars tested. Besides, it was shown that the HTC phenomenon affected the antioxidant activity of beans which is a potential and nutritional characteristic of beans. This phenomenon is still a concern for developing countries because the extended cooking time requires the use of more energy for processing, and the HTC beans have lower nutritional value than normal edible beans.

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Supporting Information Available: ANOVA tables from the analysis of the content of phenolic acids and Trolox equivalent antioxidant capacity. This material is available free of charge via the Internet at http://pubs.acs.org.

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